SYNOPSIS

MICROPROPAGATION STUDIES AND COMPARATIVE PHARMACOGNOSTICAL PROFILE OF *in vitro* AND *in vivo* TISSUES OF *Withania somnifera* (L.) POSHITA VARIETY

SUBMITTED BY

VIJI, M.O.

SUPERVISOR

DR. R. PARVATHAM

SUBMITTED TO

AVINASHILINGAM INSTITUTE FOR HOME SCIENCE AND HIGHER EDUCATION FOR WOMEN, COIMBATORE – 641 043

INDIA

JULY 2011

Introduction

Medicinal plants are the richest bioresource in traditional systems of medicines, modern medicines, pharmaceutical intermediates, folk medicines and chemical entities for synthetic drugs (Hammer *et al.,* 1999). These synthetic drugs are derived either from whole plants or from different plant parts like leaves, stems, barks, roots, flowers and seeds or from excretory plant products such as gum, resins and latex. Some important chemical intermediates needed for manufacturing modern drugs are also obtained from plants eg. diosgenin and solasodine. The plant based drugs are reported to have fewer side effects when compared with synthetic antibiotics (Vinod *et al*., 2010). The present enthusiasm in the search for natural sources of biologically active compounds from plants has all the more enhanced the importance of medicinal plants.

Withania somnifera (L.), also known as 'Ashwagandha', is an important medicinal plant, which is cultivated in India for its medicinal purposes. The roots and leaves of Ashwagandha contain various alkaloids, viz., withanolides (Atta *et al.,* 1991) and withaferins (Devi *et al.,* 2000). The withanolides are steroidal compounds and bear resemblance, both in action and appearance to the active ginsenosides of Asian Ginseng. Studies show that the plant has been used as an antioxidant, adaptogen, aphrodisiac, liver tonic, anti-inflammatory agent, antitumour, astringent and more recently to treat ulcers, bacterial infection, venom toxins and senile dementia. Clinical trials and animal research support the use of the plant for anxiety, cognitive and neurological disorders, inflammation, hyperlipidemia and Parkinson's disease (Gupta and Rana 2007).

The metabolic constituents, particularly the secondary metabolites differ with the variety of *W. somnifera* (L.), tissue type and sometimes with growth conditions (Abraham *et al.,* 1968*).* Also, depending upon the season, weather conditions and infection, the content of withanolides vary. Such variations often lead to inconsistent therapeutic and health promoting properties of various commercial *Withania* preparations (Sangwan *et al.,* 2004; Dhar *et al.,* 2006).

Since ancient times, the root has mostly been obtained from the wild plant. But, nowadays, most of the roots available in the Indian market are obtained from the cultivated plants. Improved varieties are preferred in the drug formulation owing to the absence of tongue paralyzing agents, which are common in the wild population of the plant (Kaul, 1957). Cultivated plants show many different chemotypes (Kirson *et al.,*1971) and Poshita, one such variety with improved chemical quality had been released by the Central Institute for Medicinal and Aromatic Plants, Lucknow, India (Misra *et al.,* 2001). Kumar *et al.,* (2007*)* reported that among 25 accessions of *Withania somnifera* (L.)*,* the morphometric and root yield attributes show higher values in wild accessions as compared to the cultivated ones, whereas, superiority in terms of withaferin A content is exhibited by cultivated accessions.

The species is commercially propagated by seeds because of the lack of natural ability for vegetative propagation (Sen and Sharma, 1991). Its propagation through seed is very difficult due to high dormancy and low germination percentage. Also the seed viability is limited to one year (Rani and Grover 1999), making the long duration seed storage futile. Further, under field conditions, seed rot and blight in the young seedlings stage are the common diseases which reduce plant population and ultimately root yield (Farooqi and Sreeramu, 2004).

Report is also available for the withanolide production and isolation in morphologically differentiated cultures such as undifferentiated callus, organogenic callus, multiple shoots and whole *in vitro* plantlet of wild variety (Sharada *et al.,* 2007). Suspension cultures of *Withania somnifera* (L.) cells were established and shown to produce withaferin A. In the native plant, withaferin A represents a very minor proportion of withanolide component. Investigation of tissue culture of plants or its parts may lead to the development of new plant based drugs. Therefore, it is of great interest to optimize the conditions for the production of *Withania* plants and to carry out the withanolide isolation in order to validate their use in modern medicine.

Hence, the study was formulated with the following objectives:

☛ To standardize a reproducible protocol for rapid multiplication of *Withania somnifera* (L.) Poshita variety under *in vivo* and *in vitro* conditions.

☛ To encompass the pharmacognostical studies of *Withania somnifera* (L.) which include preliminary phytochemical screening and quantification and localize some of the primary metabolites by histochemical studies.

☛ To assess the antioxidant, cytotoxic, antibacterial and antifungal activities of the plant extracts.

☛ To isolate the important phytochemical compounds from the plant extracts, identify and characterize them by using standard analytical methods, such as TLC, UV and FT-IR.

The work was carried out in four phases.

Phase I involves the standardization of a technology for multiplication of the plant *in vivo* and *in vitro*. *In vivo* multiplication was tried with seeds and vegetative propagules. *In vitro* multiplication was conducted through micropropagation technology.

Phase II of the research was to analyze the phytochemicals, from the crude extracts of leaf, stem and root of *in vitro* and *in vivo* propagated plants.

Phase III of the study was formulated to assess the pharmacological effects of *Withania somnifera* (L.) Poshita variety, namely, antioxidant, antibacterial, antifungal and cytotoxic activity of both *in vivo* and *in vitro* grown plants.

In Phase IV, the major secondary metabolites were isolated and identified.

Materials and methods

Phase I

Ashwagandha seeds (*Withania somnifera* (L.) Poshita variety) were collected from the Central Institute of Medicinal Aromatic Plants (CIMAP), Lucknow. Hundred seeds of the Poshita variety was soaked in water for 24 hours, 48 hours and 72 hours and then sowed on soil. Germination percent of the seeds were noted. For *in vitro* germination, the surface sterilized soaked seeds were inoculated into bottles containing MS basal medium with 1 to 6% of sucrose concentration.

Direct organogenesis

Shoot tip explants with tip meristems and four to five nodes and 8-10 mm in length were excised from *in vitro* raised seedlings and directly transferred to the surface of the medium containing 0.05 to 0.5mg l^{-1} of TDZ and 0.5 to 5.0 mg l^{-1} of BAP. Individual shoots were isolated and transferred to the elongation medium containing KIN

(0.2 to 0.1 mg l^{-1}) and /or GA_3 (0.2-1.0 mg $l^{-1)}$, supplemented with 1.0 mg l^{-1} BAP for elongation and flowering. The elongated shoots were excised and transferred to MS basal medium for rooting. Rooted plantlets were kept for hardening. The pollen viability was determined by hemocytometer method.

Indirect organogenesis

The leaf segment explants were inoculated on MS medium containing 2, 4-D (0.5 to 1.5mg l^{-1}) with or without KIN (1.0 to 3.0mg l^{-1}). Similarly, trials were also conducted with media containing (0.1 to 0.3mg l^{-1}) TDZ with or without (2mg l^{-1}) BAP. Regenerated multiple shoots were sub cultured to shoot elongation medium containing 1mg l^{-1}BAP. Individual shoots were isolated from the multiple shoots and transferred to MSO medium. The rooted plants kept for hardening.

Leaf segments were excised from the seedlings maintained by *in vitro* germination of seeds and cultured on MS medium supplemented with IBA (1 to 5mg l^{-1}), IAA (0.2 to 1.0mg l^{-1}) and NAA (0.2 to 1.0 mg l^{-1}) individually or in combination for adventitious root formation.

Phase II

Preliminary screening and quantitative estimation of phytoconstituents in *in vivo* and *in vitro* regenerated leaf, stem and root of *Withania somnifera* (L.) Poshita variety were carried out. The carbohydrate and protein contents of the plant parts of *Withania somnifera* (L.) Poshita variety were assessed.

The powdered samples of three month old and five month old roots, stems and leaves of *in vitro* and field grown *Withania sominfera* (L.) Poshita were serially extracted with solvents of increasing polarity, namely, chloroform, ethyl acetate and methanol and subjected to TLC. LC-MS analysis of crude methanol extract of leaf callus and *in vitro* and *in vivo* propagated leaf were also carried out.

Phase III

The activities of various enzymic and non enzymic antioxidants were analysed. The crude extracts of leaf, stem and root were assessed for their radical quenching ability against a battery of radicals such as ABTS, DPPH, H_2O_2, OH, SO and NO radicals. Antibacterial screening tests with crude extracts of the dried leaf, stem and root in *in vivo* and *in vitro* propagated plants were conducted against *Bacillus subtilis*,

Escherichia coli, *Staphylococcus aureus*, *Shigella flexneri*, and *Klebsiella pneumonia.* The activities of the *in vitro* and *in vivo* propagated plant parts against five fungal cultures, namely, *Aspergillus flavus*, *Candida albicans*, *Aspergillus niger*, *Mucor indicus and Rhizopus stolonifer* were tested. The lethality of various extracts(chloroform, ethyl acetate and methanol) of *in vitro* and *in vivo* propagated leaf, shoot and root of *Withania somnifera* (L.) Poshita variety to brine shrimp (*Artemia salina*) was determined after 24 hours of exposure.

Phase IV

The dried in *vitro* propagated leaf material powder was extracted with methanol and loaded on a silica gel column and eluted with different solvents. The fractions were tested by TLC. Those fractions with matching profiles were pooled, concentrated and further purified by column. The purified fractions were subjected to spectral analysis based on UV, IR and HPLC.

Results

Phase I

***In vivo* germination**

In *in vivo* germination, seeds soaked for 24 hours in water resulted in 65 per cent germination in one month, whereas seeds soaked for 48 hours in water did not show any further increase. However 72 hours soaked seeds exhibited 100 per cent germination within 3 days.

***In vitro* germination**

In *in vitro* germination, seeds soaked in water for 24 hours and inoculated on half strength MS medium containing various sucrose concentrations, the medium with 2% sucrose was found to give the highest germination when compared with other concentrations. However, 100 per cent germination was obtained in seeds soaked for 72 hours and inoculated in 3% sucrose within 3 days.

Direct organogenesis

Among the various concentrations of phytohormones, TDZ (0.1mg l^{-1}) and BAP (1mg l^{-1}) showed significant increase in multiplication. In combinations of 1mg l^{-1} BAP with 0.05 to 0.5 mg l^{-1} TDZ, significant ($p>0.05$) increase in the number of shoots was found with1mg l^{-1} BAP and 0.1mg l^{-1} TDZ. For elongation, different combinations of

plant growth regulators such as BAP, TDZ, and KIN were tested. BAP (1mg l^{-1}) and GA_3 (0.4mg l^{-1}) showed significant ($p>0.05$) increase in elongation within 15 days of subculture when compared to other combinations and concentrations. Two different patterns of responses were observed depending upon the hormones in the subculture medium. The medium with BAP and GA_3 induced elongation of shoots, while BAP and KIN promoted flowering. No flowering was observed in the other combinations of BAP and KIN.

The pollen viability of *in vitro* regenerated plants was found to be reduced on comparison with that of the wild variety.

Indirect organogenesis

In different combinations of hormones with MS medium, the one with 0.2mg l^{-1} TDZ and 2mg l^{-1} BAP proliferated on the same medium and regenerated roots and shoots from the callus. Regenerated multiple shoots were subcultured on shoot elongation medium containing 1mg l^{-1} BAP. The callus obtained was sub cultured and the regenerated plants were uprooted and transferred to the field.

Histological study of callus showed the presence of several groups of meristematic cells inside the callus in the early stage. Apart from this, large number of meristems from the surface of the callus at a later stage clearly shows the possibility of producing large number of shoots from the callus of the Poshita variety.

IBA (2mg l^{-1}) and IAA (0.4mg l^{-1}) were found to be the best for root induction with the maximum number of healthy roots.

Phase II

Preliminary screening results showed the presence of phytochemical constituents such as alkaloids, anthroquinones, flavonoids, phenols, reducing sugars, saponins, phytosteroids and tannins, glycosides and terpenoids in leaf, stem and roots *in vivo* and *in vitro* propagated plants. The histochemical analysis showed that the presence of these secondary metabolites was more in *in vitro* regenerated plant tissues compared to *in vivo* tissues.

Phenols, tannins, reducing sugars, chlorophyll and alkaloids of *in vitro* leaves were found to be significantly higher ($p>0.05$) when compared with other *in vitro* and *in vivo* propagated plant parts. The carbohydrate and protein contents were found to be significantly higher in leaf samples of both *in vitro* and *in vivo* grown plants when compared to shoot and root.

Comparison of R_f value of the compounds in *in vivo* and *in vitro* propagated three month old leaf, stem and root with that of the standards (Withaferin A and Withanolide A) showed the presence of withaferin A in all the three extracts, whereas, in *in vivo* and *in vitro* propagated five month old leaf, stem and root showed the presence of withaferin A and withanolide A in all the three samples.

LC-MS analysis of crude methanol extract of leaf callus showed the presence of 20 compounds. Among them, 9 compounds were identified as amino butyric acid, pipecolic acid, methoxy cinnamaldehyde, ascorbic acid, vasicinone, taxol, epicatechin, ergosterol, and rutin. Remaining 11 compounds are yet to be identified. LC-MS analysis of crude methanol extract of *in vitro* propagated leaf showed the presence of 23 compounds. Among them, 7 compounds were identified as taxifolin, arachidic acid, cyanoalanine, scopoletin, alpha linolenic acid, tigloidine and withaferin A. The remaining 16 compounds are yet to be identified. LC-MS analysis of crude methanol extract of *in vivo* propagated leaf showed the presence of 18 compounds. Among them, 6 compounds were identified as coumestrol, diosmetin, oleic acid, arachidic acid, mannose and β-amyrin. The remaining 12 compounds are yet to be identified.

Phase III

The activities of the enzymic antioxidants superoxide dismutase and catalase were significantly high ($p>0.05$) in roots in both *in vitro* and *in vivo* plant parts. The *in vivo* and *in vitro* propagated leaf showed maximum activity in peroxidase, glutathione peroxidase and glutathione-S-transferase. The activities of the non-enzymic antioxidants, namely, ascorbic acid, α-tocopherol, carotenoids, polyphenols and flavonoids were significantly higher ($p>0.05$) in leaves of both *in vitro* and *in vivo* propagated plants.

Among the three extracts assessed the maximum scavenging effect was elicited by the methanolic extract of in vitro regenerated leaves showed a powerful scavenging against a group of radicals such as DPPH, ABTS, H_2O_2, OH radicals, SO radicals and NO radicals followed by the chloroform extract and the least scavenging effect was from the ethyl acetate extract.

Among chloroform, ethyl acetate and methanol extracts of *in vitro* leaf, stem and root samples, ethyl acetate extract of leaf exhibited significantly higher ($p<0.050$) inhibitory activity against *Bacillus subtilis*, *Escherichia coli*, and *Shigella flexneri*. Ethyl acetate of root extract showed maximum inhibitory zone against *Staphylococcus aureus* and *Klebsiella pneumoniae*. In the tests with crude extracts of *in vivo* propagated plant parts, with regard to leaf, stem and root, the chloroform extracts of leaf exhibited highest inhibition against *Escherichia coli*, *Shigella flexneri* and *Bacillus subtilis* respectively and chloroform extract of the stem exhibited highest inhibition against *Klebsiella pneumoniae* and *Staphylococcus aureus.*

The methanol extract of the leaf exhibited maximum inhibition against all the fungal strains when compared with stem and root of *in vitro* and *in vivo* propagated plants.

In brine shrimp lethality test the root exhibited greater cytotoxic effect when compared to leaf and shoot. *In vitro* propagated root extracts showed higher activities when compared to *in vivo* propagated root. The cytotoxicity exhibited by the crude extracts was promising and this clearly indicates the presence of potent bioactive compounds in *Withania somnifera* (L.) Poshita variety.

Phase IV

Structural analysis of the isolated compound was confirmed as withaferin A.

Conclusion

In the present study, a standardized protocol was developed for micropropagation of *Withania somnifera* (L.) Poshita variety through direct and indirect regeneration method. The preliminary phytochemical screening indicated the presence of secondary metabolites in *in vivo* and *in vitro* plant parts. The identification of withanolides in the callus as well as *in vitro* induced adventitious roots points to the possibility of using *in vitro* tissue culture as an alternative source for production of

secondary metabolites. LC-MS analysis of crude samples showed the presence of many phytochemical compounds. *Withania somnifera* (L.) Poshita variety exhibited resistance against the tested bacterial and fungal strains and also possessed a good cytotoxic and antioxidant effect in *in vitro* propagated plant parts. Methanol extract showed the presence of Withaferin A as the major compound.

References

Abraham, A., Kirson, I., Glotter, E. and Lavie, D.A., (1968), Chemotaxonomic study of *Withania somnifera* (L.) dunal, Phytochemistry, 7: 957–962.

Atta, U. R., Jamal, S.A., Choudhary, M.I. and Asif, E. (1991), Two withanolides from *Withania somnifera* (L.), Phytochemistry, 30:3824–3826.

Devi, P.U., Kamath, R. and Rao, B.S.S. (2000), Radio sensitization of a mouse melanoma by withaferin A: *in vitro* studies, Ind. J. Exp. Biol., 38:432–437.

Dhar, R.S., Verma, V., Suri, K.A., Sangwan, R. S., Satti. N. K., Kumar, A., Tuli, R. and Qazi, G.N. (2006), Phytochemical and Genetic Analysis in Selected Chemotypes of *Withania somnifera* (L.), Phytochemistry, 67: 2269-2276.

Farroqi, A. A. and Sreeramu, B. S. (2004), Cultivation of medicinal plants, University Press, India.

Gupta, G.L. and Rana, A.C. (2007), *Withania somnifera* (L.) (Ashwagandha): a review, Pharmacog. Rev., 1:129–136.

Hammer, K.A., Carson, C.T. and Riley, T.V. (1999), Antimicrobial activity of essential oils and other plant extracts, J. Appl. Microbiol., 86 (6): 985-988.

Kaul, K.N. (1957), On the origin, distribution and cultivation of Aswagandha, the so called *Withania somnifera* (L.) Duanal, of Indian literature on material medica, Pharmaceutical and drug committee symp. on the utilization of the Indian medicinal plants, Lucknow,12-14 p.7-8.

Kirson, I., Glotter, E., Lavie, D. and Abraham, A. (1971), Constituents of *Withania somnifera* (L.) Dun part XII the withanolides of an Indian chemotype. J. Chem. Soc. C., 2032-2044.

Kumar, A., Kaul, M.K., Bhan, M.K., Khanna, P.K. and Suri, K.A. (2007), *Withania somnifera* (L.) Dunal (Solanaceae). Genet. Resour. Crop Evol., 54: 655–660.

Misra, H.O., Sharma, J.R., Lal, R., Gupta, M. M., Shunkla, N., Singh, N.K. and Singh, N. (2001), Registration of new variety Poshita of *Withania somnifera* , J. Med. Arom. Pl. Sci., 23:97-98.

Rani, G. and Grover, I.S. (1999), *In vitro* callus induction and regeneration studies in *Withania somnifera* (L.), Plant cell. Tiss. Org. Cult., 57:23-27.

Sen, J. and Sharma, A. K. (1991), Micropropagation of *Withania somnifera* (L.) from germinating seeds and shoot tips, Plant cell. Tiss. Org. Cult., 26:71-73.

Sharada, M., Ahuja, A., Suri, K. A., Vij, S. P., Khajuria, R. K., Verma, V. and Kumar, A. (2007), Withanolide production by *in vitro* cultures of *Withania somnifera* (L.) and its association with differentiation, Biologia Plantarum, 51(1): 161-164.

Sharma, V., Gupta, A.P., Bhandari, P., Gupta, R. and Singh, B. (2007), A Validated and Densitometric HPLC Method for the Quantification of Withaferin- A and Withanolide-A in Different Plant Parts of Two Morphotypes of *Withania somnifera* (L.), Chromatographia, 66: 801 – 804.

Sangwan, R.S., Chaurasia, N.D., Misra, L.N., Lal, P., Uniyal, G.C., Sharma R., Sangwan N.S., Suri. K. A., Qazi. G. N. and Tuli, R. (2004), Phytochemical variability in commercial herbal products and preparaations of *Withania somnifera* (L.) (Ashwagandha), Curr. Sci., 86:461–5.

Vinod, N.V., Haridas, M. and Sadasivan, C. (2010), Isolation of 1,4-naphthalenedione, an Antibacterial Principle from Leaves of *Holoptelea integrifolia* and its Activity Against β-lactum Resistant *Staphyloccocus aureus*, Ind. J. of Biochemistry and Biophysics, 47: 53-55.